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First Named Inventor	:	Wank, Rudolf		
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Title	:	Use of Stimulated Peripheral-Blood Mononuclear Cells for the Treatment of Cancerous Diseases		

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Rudolf Wank, hereby declare as follows:

1. I am a citizen of Germany, residing at Muenchen, Hans-Sachs-Str.12,

80469 Germany.

2. I received the Medical license from the University of Muenchen 1968, the degree as scientist of immunology (Dr.med.habil) 1987. I was employed as research fellow at the University of Muenchen (1973-1.74), as research associate at the University of Madison/Wisconsin (2.1974-1975), as research associate/associate at the Sloan Kettering Institute for Cancer Research, New York, NY (1976-3.1979). I was appointed as member of the Institute of Immunology, University of Muenchen, 1979 and was employed there as Professor of Immunology until 2006. Since 2007 I direct a medical office for cellular immunotherapy. I am Head of the Immune Therapy Research Center Immunis e.V

3. I am an inventor of U.S. Patent Application No. 10/687,913.

4. I am familiar with the Office Actions of January 7, 2009 and December 3, 2007, as well as with the art cited therein. I understand that claims 29-32, 34, 35, 40, 41, 43 and 44 stand rejected under 35 U.S.C. § 103(a) as purportedly unpatentable over Babbitt et al. (U.S. Patent No. 5,766,920) in view of Gold et al. (*J Surg Re.* 1995 Aug;59(2):279-86), Rudolf Wank (International Publication No. WO 99/50393) and Marzo et al. *(J Immunol* 1999 May 15;162(10):5838-45). I also understand that claims 29 and 33 stand rejected under 35 U.S.C. § 103(a) as purportedly unpatentable over Babbitt view of Gold, Wank, Marzo, and in further view of Gale Granger (U.S. Patent No. 5,837,233) and Johnson et al. (U.S. Patent No. 5,217,704). However, the methods and CD3-activated cells disclosed in the cited references are very different from the methods and CAPRI cells of the present invention. This assertion is supported by the data set forth herein, which shows that CAPRI cells are far more effective in killing cancer cells than standard CD3-activated cells, such as the CD3-activated cells used in the methods of the cited references, including Babbitt and Gold.

5. The present invention provides a cascade priming (CAPRI) method which effectively destroys malignant tumor cells. The cells produced using this method are far more cytotoxic to malignant cells than conventional CD3-activated cells. The CAPRI method enhances HLA expression in cancer cells of different types, which is crucial for MHC-restricted lysis. Thus, the CAPRI method can be useful against many types of cancers.

6. The CAPRI method is able to stimulate antigen presenting cells to express and then to transfer tumor antigen information into naïve T cells, which causes the naïve T cells to differentiate into T effector cells. Unstimulated, autologous PBMC are used as a source of

naïve T cells, which respond to the information regarding the tumor provided by the antigen presenting cells. The active immune cells used in this method are called CAPRI cells.

7. The ability of the CAPRI method to cause cancer cell death results from the cooperation of four types of cells: T helper cells, T cytotoxic cells, dendritic cells, and monocytes. Monocytes and dendritic cells are stimulated by CD3-activation of T cells. Next, unstimulated T cells are added, to address the known issue of CD3-activated T cells internalizing antigen-specific T cell receptors. The efficacy of this method as compared to known methods using CD3-activated cells is discussed below.

8 Attached herewith is a manuscript by myself , B.Laumbacher and S.Gu showing the efficacy of CAPRI cells against cancer cells in comparison to conventional CD3-activated cells, such as those cells used in Babbitt and Gold. At best, CD3-activated PBMC were shown to only cause a minimal amount of cancer cell death. In contrast, the CAPRI method surprisingly caused nearly total lysis of autologous cancer cells. As discussed on pages 3-4 of the manuscript, after 18-24 hours of treatment, CAPRI cells successfully lysed almost all of the autologous cancer cells. In contrast, coculture of cancer cells with CD3-activated cells showed little or no lysis of cancer cells.

10. With the CAPRI method, when stimulation occurs via cascade priming, the first PBMC population is activated with anti-CD3 antibodies. This PBMC population becomes the primary stimulated PBMC with internalized CD3 chains and internalized $\alpha\beta$ TCR. Because of this internalization, the primary stimulated PBMC can no longer be differentiated into effector cells. However, the T cells of the primary stimulated PBMC do produce cytokines, which stimulate antigen-presenting cells. Once the primary stimulated

PBMC are incubated with naïve PBMC, the stimulated antigen presenting cells provide immunogenic cancer peptides to the $\alpha\beta$ TCR of the non-stimulated T cells in the naïve PBMC. The $\alpha\beta$ TCR stimulation results in differentiation naïve T cells into cytotoxic and helper effector T cells, which kill cancer cells. As shown in Figure 1, attached, the T cells from the primary stimulated PBMC are not active on the cancer cells because they have internalized the $\alpha\beta$ TCR.

11. The CAPRI method also provides other advantages over known methods. The CAPRI method produces cytotoxic effector T cells which lyse cancer cells in a MHC-restricted manner (see Figure 2, attached). In addition, the CAPRI method causes the maturation of freshly added $CD14^+$ monocytes that are included in the naïve PBMC into $CD83^+$ dendritic cells (see Figure 4, attached). Furthermore, CAPRI cells enhance the HLA class I and HLA class II surface expression in epithelial and other solid malignant tumor cells. This increases the expression of immunogenic tumor peptides, which is crucial to the high cytotoxic capacity of CAPRI cells against various cancer cells (see Figure 3, attached).

It is my opinion that the references cited in the present Office Action do not describe the claimed CAPRI method as set forth above. The primary reference, Babbitt, uses an activation process which is very different from the CAPRI process of the presently claimed invention. Only activation of T cells by way of the $\alpha\beta$ TCR causes the differentiation of the T cells into MHC-restricted effector T cells. Activation with immobilized anti-CD3 antibodies causes internalization of both CD3 and $\alpha\beta$ TCR of the T cells (see the attached manuscript at page 4, lines 3-6). Furthermore, the CD3-activated cells of Babbitt and Gold are not effective at successfully lysing cancer cells, where in contrast the attached data show that CAPRI cells are effective in killing cancer cells.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code.

Respectfully Submitted,

Date: May 15, 2009

Rudolf WANK

Attachment

CURRICULUM VITAE

Prof. Dr.med. Rudolf Wank

I. PERSONAL

Date of birth: September 23, 1939

Place of birth: Prag, Czchoslovakia

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II EDUCATION

University Muenchen Germany	1959-1965
Internship:	
Orthopedics, University Muenchen	10-11.1965
Pathology, Hospital r.d.Isar, Technical University Muenchen	12.65-6.1966
Internal Medicine, Surgery, Gynecology Hospital Haag/Obb.	6.66- 6.1967
Hospital Bad Wörrishofen Germany	11.67-4.1968
Postgraduate:	
Pathology, Academic Hospital Muenchen- Schwabing, University of Muenchen	1971-1972

III PROFESSIONAL QUALIFICATIONS

Medical license (Arzt) Bavarian Ministry, Germany	4.1968
Doctor of Medicine Degree Faculty of Medicine University Muenchen	6.1971
Dr.med.habil. *theme:* *The immunological discrimination of self and non-self*	1.1987

IV MEDICAL APPOINTMENTS

Internal Medicine Hospital Bad Wörrishofen, Germany	4.-8.1968

(IV)

General Medicine — 8.68-1970
Hospital Wolnzach, Germany

Pathology, Academic Hospital
München-Schwabing, University Muenchen — 1.71-12.1972

V RESEARCH APPOINTMENTS

Research fellow — 1.73-01.1974
HLA Immunogenetics Laboratory
University of Muenchen, Germany

Areas of research:
transplantation antigens defined
by antibodies, HLA associations with disease

Research Associate — 2.74-12.1975
Immunobiology Research Center
University of Wisconsin

Areas of research:
transplantation antigens defined
by primed lymphocyte typing (PLT)

Research Associate — 1.76-11.1978
Sloan Kettering Insitute for
Cancer Research
New York, New York, USA

Associate — 12.78-3.1979
Sloan Kettering Institute for
Cancer Research
New York, New York, USA

Areas of research:
immune response genes defined
by primed lymphocyte typing

Scientific Assistant, Member — 4.79- 1.1987
Institute of Immunology
University of Munich, Germany

Privat Dozent of Immunology — 1.87- 6.1993
Institute of Immunology
University of Munich, Germany

Areas of research in Munich next page

Prof. of Immunology 6.93- 2006
Institute of Immunology
University of Munich, Germany

Areas of research:
Immune response genes defined by T cell clones and HLA genetics; genes involved in processing (LMP) and transportation (TAP) of bacterial, viral and tumor antigens, intracellular bacteria and basis of psychiatric diseases

Head of Immunis e.V. since 2007
Immune Therapy Research Center

Areas of research:
Immune response genes, LMP and TAPgenes and bacterial, viral and tumor antigens, intracellular bacteria and psychiatric diseases adoptive immune cell therapy

VI SOCIETIES

Medical Association of Bavaria

American Association of Histocompatibility and Immunogenetics

German Society of Immunology

German Society of Immunogenetics

VII AWARDS

Schultz Prize for Research in Sqamous Cell Carcinoma

Science Prize of the German Society of Immunogenetics for the cascade priming (CAPRI) method

CANCER CELL DESTRUCTION IN VITRO AND IN PATIENTS BY LYMPHOCYTES PRIMED WITHOUT TUMOR CELLS OR IDENTIFIED TUMOR PEPTIDES

Until now adoptive immunotherapy has recquired definition of tumor-specific immunogenic material. Using immune cells from the peripheral blood we achieved with a novel method of activation, that a cellular quartett of monocytes, dendritic cells, T helper and T cytotoxic cells lysed efficiently different autologous cancer cells in vitro. Cell primed with this novel method increased significantly five year survival rates of patients in an adjuvant intention-to-treat setting. We first stimulated monocytes and dendritic cells by CD3-activation of T cells. In the next step we added unstimulated T cells since CD3-activated T cells internalize the antigen-specific T cell receptor. $CD14^+$ monocytes, indispensable for the cascade priming (CAPRI) process like T cells, showed after 24 hours increased maturation of $CD14^+$ monocytes to $CD1a^+$, $CD83^+$ dendritic cells in CAPRI cultures, numbers of $CD4^+$ $CD25^{high}$ $FOXp3^+$ regulating cells decreased. CAPRI cells enhanced HLA class I and class II expression in cocultured cancer cells of different types, crucial for MHC-restricted lysis by CAPRI helper and cytotoxic T lymphocytes. In vitro results indicate that the CAPRI method can be used for many cancers.

Introduction

Numerous therapeutical modalities have been developed to hinder growth or to induce destruction of malignant tumor cells. The multitude of modalities reflects the inexhaustible number of strategies used by cancer cells to evade control by immune cells. But the immune system seems to be able to develop counter strategies. For example, not all women infected with cancer-associated human papilloma viruses (HPV) develop carcinomas. Women with a certain immunogenetic endowment, i.e., with certain HLA alleles, are at high risk, whereas women with other HLA alleles are at low risk to develop this cancer[1,2]. HLA alleles and the encounter with a certain HPV type were decisive for development of cervical cancer[3,4] . Unrecognized immune responses must prevent the rise of carcinoma cells in women carrying resistance-associated immune response genes of the HLA system[5]. Immune surveillance of cancer growth by T lymphocytes necessarily includes recognition of tumor-immunogenic peptides. To induce such T cells, recent strategies have focussed on the use of dendritic cells that have been incubated in vitro with tumor cell lysates, pulsed with defined tumor peptides or transfected with RNA or DNA from tumor cells[6,7].

Gene mutations and corresponding mutated cellular proteins can be recognized as "tumor markers" in early stages of cancer development. For example, mutations of the p53 gene have been identified as free circulating DNA in pre-cancer and cancer patients[8,9]. Cytotoxic T cell responses to different and differently mutated tumor targets have been reported[10-16]. We have been interested to identify conditions, which would stimulate antigen presenting cells (APC) to process, express and transfer tumor antigen information to naïve T cells, leading to their differentiation into T effector cells and preventing their inactivation or deletion by cancer cells, as observed in tumor infiltrating lymphocytes[17,18].

We decided to activate APC by activating T cells in PBMC cultures with the monoclonal antibody OKT3 for the following reasons: activated T cells produce a wealth of cytokines such as the monocyte-activating interferon γ (IFNγ), which stimulates intracellular processing as well as strong expression of the pMHC and of costimulatory molecules. Activated APC, however, may not be able to deliver the tumor-immunogenic message to CD3-activated T cells, since ligation of CD3 chains by OKT3 antibodies induces internalization of the CD3/TCR complex[19,20]. We therefore added to the CD3-activated cells unstimulated autologous PBMC as a source of naïve T cells which could respond to the tumor-specific information from the activated APC. This approach induced a cascade of events leading to the priming of naïve T cells. We coined the procedure cascade priming (CAPRI) and the actively involved immune cells CAPRI cells.

Here we show that CAPRI cells are much more effective in the destruction of malignant tumor cells then are CD3-activated cells and this cytotoxicity is MHC restricted. Cascade priming induced differentiation of monocytes to dendritic cells and enhanced expression of costimulatory molecules. In addition CAPRI cells lead to increased MHC class I and MHC class II expression by malignant tumor cells, thus allowing both CD4 and CD8 T effector cells to recognize the cancer cells. We show that the optimal cancer cell lysis observed with CAPRI cells results from the cooperation of a cellular quartett consisting of T helper cells, T cytotoxic cells, dendritic cells and monocytes.

RESULTS

Nearly complete lysis of autologous cancer cells by CAPRI cells but not by CD3-activated cells after 18-24 hours.

We stimulated APC in PBMC bulk cultures by activating T cells with the OKT3-CD3 antibody, which binds to a conformational epitope expressed either by CD3-εδ or CD3-εγ chains[22]. Activated T cells produce monocyte activating cytokines such as IFNγ which can lead to activation of APC. Since ligation of CD3 molecules downmodulates the CD3:αβTCR complex by internalization or by prevention of recycling[19,20] (not shown), we added autologous unstimulated PBMC to CD3-activated PBMC to provide a source of naïve T cells which can respond to the APC in a procedure we called cascade priming (CAPRI). After one day in culture CAPRI cells were compared with CD3-activated cells for their ability to kill autologous cancer cells.

The destruction of cancer cells by CAPRI and by CD3-activated cells was measured by microscopic inspection (**Fig. 1a,b,c,d,e,f**) and by the chromium[51] release assay (**Fig. 1g**). Figure 1 shows an example of lysis of breast carcinoma cells. Microscopic inspection of the tumor cells indicated no or little reduction of cancer cell numbers after 20h coculture with CD3-activated cells (**Fig. 1e**) compared with carcinoma cultures at time 0 (**Fig. 1a,b**), whereas lysis of most cancer cells was observed after 20h of coculture with CAPRI cells (**Fig. 1f**). In chromium[51] release assays CD3-activated PBMC showed no significant lytic activity (**Fig. 1g**), while CAPRI cells lysed 27.1% of cancer cells at a 5 :1 effector to target (E:T) ratio and 89.9% of cancer cells at a E:T ratio of 20:1 (**Fig. 1g**). Furthermore, CAPRI cells could be successfully reseeded onto new cancer cell lawns without addition of cytokines (not shown). Similar results were observed in several different patients with different malignant tumor types (**Table 1**)

HLA class I and HLA class II antibodies abrogated lysis of cancer cells and lysis of allogeneic cancer cells required HLA sharing.

Generation of cytotoxic T cells depends on interactions between the αβ TCR and the pMHC[24]. MHC-restricted interactions were analysed using allogeneic cells and antibodies blocking the pMHC complex. Figure 3 shows results of CAPRI cells from two unrelated breast cancer patients with defined HLA-DQ class II alleles. These cells were cocultured with breast cancer cells from 6 unrelated female patients (**Fig. 2a**). The degree of lysis was estimated microscopically after 24h of coculture using the HLA microcytotoxicity scale (see materials and methods). CAPRI cells lysed autologous cancer cells strongly and allogeneic cancer cells with shared HLA-DQ alleles approximately half as well, whereas lack of HLA-DQ sharing resulted in only minimal background lysis (**Fig. 2a**). This suggested that HLA class II surface molecules of APC presented tumor-immunogenic peptides, but complete lysis may depend on sharing of both HLA class I and HLA class II antigens. This was indirectly supported by blocking lysis of autologous cancer cells with antibodies against HLA class I and class II molecules. Lysis of cancer cells by CAPRI cells (**Fig. 2c**) was strongly diminished either with the antibody W6/32 binding to all HLA class I molecules (**Fig. 2e**) or with the antibody CIA2 binding to the HLA-DQ class II molecules (**Fig. 2d**). The required concurrence of HLA class I and class II presentation indicated a comprehensive interdependence of helper and cytotoxic T cells for successful lysis of cancer cells. As expected, CAPRI cells showed only very weak activity against the NK target cell K562, which usually does not express HLA antigens (not shown). This very weak lytic activity may come from NKT cells in the PBMC cultures[25].

CAPRI cells enhanced HLA class I and HLA class II surface expression in epithelial and other solid malignant tumor cells

CAPRI cells lysed cancer cells in an HLA restricted manner (**Fig. 2**). Given that cancer cells tend to downregulate HLA expression to evade recognition by cytotoxic T cells[26,27]. We questioned whether CAPRI cells might upregulate the HLA expression by cancer cells. We compared the HLA expression of CFSE (5(6)-carboxy fluorescein diacetate N-succinimidyl ester) labelled cancer cells after coculture with autologous unstimulated PBMC, with CD3-activated PBMC or with CAPRI cells using a low effector to target ratio of 5:1 (**Fig. 3**). Increased mean fluorescence intensity (MFI) of gated living cancer cells could be observed only in cocultures with CAPRI cells, but not in cocultures with CD3-activated PBMC or with unstimulated PBMC (**Fig. 3**). CAPRI cell stimulated cancer cells showed a 40% increase in HLA class I expression (MFI vs MFI) and a 60% increase in HLA-DR class II expression (MFI vs MFI) (**Fig. 3a**). Enhanced MHC class II expression of cancer cells could be the deciding factor in the destructive power of CAPRI cells, since interactions between MHC class II and CD4 molecules are pivotal for the augmentation of cytotoxic T cell responses[28,29].

Maturation of monocytes to dendritic cells during the CAPRI procedure

Stimulated APC express high levels of B7 and other costimulatory molecules as well as MHC class I and MHC class II molecules[21]. We assumed that APC in PBMC bulk cultures become stimulated by CD3 activation of T cells and investigated whether differences could be observed between APC in CD3-activated PBMC and CAPRI cells. We compared phenotypic changes of monocytes during CD3-activation of PBMC with monocytes during the cascade priming procedure (**Fig. 4b**) by adding CFSE-labelled purified $CD14^+$ cells as tracer to the PBMC at day 0. Phenotypic markers of CFSE-labelled $CD14^+$ monocytes were determined before the start of activation (day 0), after one day (day 1) and after five days (day 5) (**Fig. 4**). In CAPRI

cells the monocytes lost CD14 expression and matured as defined by the acquisition of the dendritic cell markers CD1a and CD83 at day 1 and stronger at day 5 (**Fig. 4b**). Upregulation of the costimulatory molecules CD80, CD86 and CD40 as well as HLA-DR class II and HLA class I molecules was also observed (**Fig. 4b**). In CD3-activated PBMC monocytes remained $CD14^+$ and did not express CD83 and CD1a. Upregulation of costimulatory molecules CD80, CD86, CD40 as well as of HLA class I and of HLA-DR was observed but was clearly lower than in CAPRI cell cultures (**Fig. 4c, Supplementary Table 1** online). Quantitative analysis of the leucocyte subpopulations in CD3-activated PBMC and CAPRI cells from five cancer patients confirmed these observations. We found significantly increased numbers of matured dendritic cells in CAPRI cells as compared to CD3-activated PBMC (paired t-test, P=0.000096, **Supplementary Table 1** online) and a higher percentage of monocytes in CD3-activated PBMC as compared to CAPRI cells on day 5 (paired t-test, P=0.023, **Supplementary Table 1** online).

Depletion of monocytes, dendritic cells, T helper or T cytotoxic cells from PBMC or CAPRI cells reduced or forestalled T cell priming or cancer cell lysis

Depletion of $CD3^+CD8^+$ T lymphocytes, $CD3^+CD4^+$ T lymphocytes, $CD14^+$ monocytes or $CD83^+$ dendritic cells, but not of $CD19^+$ B lymphocytes (not shown) affected T cell priming or lysis of cancer cells. The influence of depletion was analysed at the following time points: 1) from unstimulated PBMC before CD3-activation, 2) from unstimulated PBMC, to be added to CD3-activated PBMC, 3) from CAPRI cells before coculture with cancer cells (**Fig. 5**).

Not unexpectedly, depletion of $CD3^+CD8^+$ T lymphocytes at each time point prevented development of any lytic capacity of CAPRI cells (**Fig. 5d**, PBMC, P_{slope} = 0.003, $P_{intercept}$ = 0.001; reimported PBMC, P_{slope} = 0.003, $P_{intercept}$ = 0.001, CAPRI

effector cells P_{slope} = 0.003, $P_{intercept}$ = 0.003. Depletion of $CD4^+$ T cells at each time point had the same effect. Thus $CD3^+CD4^+$ T cells were not only required during the priming phase but also absolutely required in the effector phase (**Fig. 5c**, PBMC, $P_{slope} = 1.25 \times 10^{-7}$, $P_{intercept}$ = 0.022; reimported PBMC $P_{slope} = 1.25 \times 10^{-7}$, $P_{intercept}$ = 0.022, CAPRI effector cells $P_{slope} = 4.6 \times 10^{-7}$, $P_{intercept}$ = 0.018. Depletion of $CD14^+$ monocytes prior to anti CD3-activation or in the PBMC added to CD3-activated cells as source of naïve T cells, completely prevented the development of lytic activity of CAPRI cells, P_{slope} = 0.003, $P_{intercept}$ = 0.001 and P_{slope} = 0.003, $P_{intercept}$ = 0.001 (**Fig. 5a**). These results are perhaps not surprising given the observation that $CD14^+$ monocytes mature into dendritic cells during the course of CAPRI cell procedure. $CD14^+$ cells were not necessary for the lytic activity of CAPRI cells (P_{slope} = 0.37, $P_{intercept}$ = 0.057).

Depletion of $CD83^+$ dendritic cells at each time point reduced the development of CAPRI lytic activity by half (PBMC, P_{slope} = 0.00072, $P_{intercept}$ = 0.008), reimported PBMC, P_{slope} = 0.004, $P_{intercept}$ = 0.007, CAPRI effector cells P_{slope} = 0.01, $P_{intercept}$ = 0.004) (**Fig. 5b**).

This indicates a continuous supply of contact-information and/or of cytokines especially by dendritic cells to T effector cells during cancer cell destruction. Maintaining a flow of information or a threshold of activating signals may prevent inactivation. This may be important for lysis of cancer cells, which do not express or express only low levels of MHC molecules or activation molecules. Failing immune responses as a consequence of rudimentary immunogenic information by cancer cells have been demonstrated[26,27].

The numbers of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes decrease during cascade priming

Several reports described regulation of immune cell homeostasis and suppression of cytolytic immune responses against human cancer cells by $CD4^+CD25^+$ regulatory T cells[30-36]. Modulation and suppression appeared antigen-specific or non antigen-specific and restricted to $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes[30-36]. We compared the percentage of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes of CD3-activated cells and of CAPRI cells. The number of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes declined only in CAPRI cell cultures (**Supplementary Fig. 1** online). This could indicate a suppressive role of some $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes. CD3-stimulation of T lymphocytes activates different pathways leading to a stronger Foxp3 expression than stimulation of the antigen TCR[37], which is addressed in the cascade priming procedure. Dendritic cells can abrogate the regulatory activity of $CD4^+CD^{25+}$ T lymphocytes of the human peripheral blood[38]. This effect could be enhanced by activated dendritic cells. Experiments will have to clarify whether a subpopulation of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes like IL-10 producing cells[36] or different $Foxp3^+$ isoforms were eliminated or neutralized during the cascade priming procedure.

Discussion

The dramatic power of autologous MHC-restricted immune responses, first recognized by Zinkernagel and Doherty[39], contrasts with the failure of MHC-restricted tumor infiltrating lymphocytes (TIL) to overcome cancer growth. One important factor of the failing immune response is likely to be the unprofessional presentation of tumor-immunogenic peptides to naïve T cells by cancer cells. To ensure professional presentation many investigators have used in vitro manipulated dendritic cells for T cell priming. Dendritic cells have been loaded with tumor antigens or transfected with

RNA encoding tumor antigens[40]. We believed that APC in the peripheral blood of cancer patients harbour sufficient tumor-immunogenic information and do not need further peptide loading or RNA transfection to prime naïve T cells against autologous cancer cells. However, we had to consider that TIL recognize tumor-immunogenic antigens but do not or not sufficiently destroy malignant tumor cells. Low level expression or lack of crucial MHC molecules as well as of costimulatory molecules may cause inactivation of TIL. To avoid these downregulatory effects and to achieve optimal expression of MHC and costimulatory molecules in all APC populations, we activated T cells with the OKT3 antibody in PBMC bulk cultures. Stimulation of APC via CD3-activated T cells resulted in the desired enhanced expression of MHC and costimulatory APC molecules (**Fig. 4**). But the message of these molecules cannot be received by CD3-activated T cells because CD3-activation downmodulates the TCR complex by internalization or by prevention of recycling[19,20]. We therefore added naïve T cells in PBMC bulk cultures to the stimulated APC. The engagement of the TCR with stimulated APC was likely a key step in generating activated effector helper and cytotoxic T cells.

The profund difference between cascade primed (CAPRI) cells and CD3-activated PBMC was shown in the lytic performance against autologous cancer cells. CAPRI cells lysed autologous cancer cells very efficiently, while CD3-activated PBMC achieved only minimal non-significant cancer cell lysis after 18-24 hours (**Fig. 1**). Three experimental results supported the notion that the tumor-immunogenic information was most likely communicated by the pMHC. First, allogeneic cancer cells were lysed by CAPRI cells only if CAPRI cells and allogeneic cells shared HLA molecules, as shown for HLA-DQB*0201 (**Fig. 2a**). Second, anti HLA class I as well as anti HLA class II antibodies strongly reduced lysis of breast cancer cells (**Fig. 2b-d**). This suggests participation of both $CD4^+$ T helper and $CD8^+$ cytotoxic T cells in

the MHC-restricted cancer lysis since HLA class I and class II antibodies blocked lysis equally efficiently. Third, lysis of the NK target K562 by CAPRI cells was minimal (not shown).

Many signalling pathways are initiated by engagement of the antigen-TCR, but full T cell activation requires costimulatory signals. We compared the expression of costimulatory molecules and the strength of MHC expression in APC of CAPRI cells compared to APC of CD3-activated PBMC by following the maturation of CFSE-labelled $CD14^+$ monocytes in both cultures (**Fig. 4**). The overall expression of costimulatory and MHC molecules increased significantly stronger in CAPRI cell cultures than in CD3-activated PBMC, but this was mainly due to the maturation of monocytes to dendritic cells in the CAPRI cell cultures. In these cultures, the numbers of CFSE-labelled $CD14^+$ monocytes decreased, whereas numbers of CFSE-labelled cells expressing the $CD1a^+CD83^+$ mature dendritic cell phenotype increased (**Fig. 4b**). In contrast, in CD3-activated PBMC as in unstimulated PBMC, numbers of $CD14^+$ monocytes did not decrease and numbers of $CD1a^+CD83^+$ dendritic cells did not increase (**Fig. 4a,c**). Paired t-tests using cells from five patients confirmed the significant difference between CD3-activated PBMC and CAPRI cells in the numbers of monocytes and dendritic cells (P=0.000096, **Supplementary Table 1** online). Furthermore, CFSE-stained cells in CAPRI cell cultures showed an increased expression of molecules which support priming of naïve T cells after one and more pronounced after five days, namely of CD40, CD80 and CD86 as well as an increased HLA expression (**Fig. 4**).

The CAPRI procedure enhanced MHC expression in APC, but carcinomas often escape recognition by downregulation of HLA expression[26,27]. We therefore examined HLA expression of CFSE-stained carcinoma cells cocultured with CAPRI cells, CD3-activated PBMC and unstimulated PBMC. Clearly, only CAPRI cells were

able to increase expression of HLA class I and class II by autologous cancer cells, either by cell contact or cytokines or both (**Fig. 3**). Upregulated HLA class I and class II expression in carcinoma cells could be a key determinant of the destructive power of CAPRI cells because both $CD4^+$ T helper and $CD8^+$ cytotoxic T cells could recognize the cancer cells.

Depletion experiments revealed an essential cooperation of a cellular quartett, consisting of $CD14^+$ monocytes, $CD14^-CD1a^+CD83^+$ dendritic cells, $CD4^+$ T helper and $CD8^+$ cytotoxic T cells, for cascade priming and for cancer cell lysis (**Fig. 5**). As expected, the presence of $CD4^+$ T helper and $CD8^+$ cytotoxic T cells was required for the development of effector cells, but the observation that both cells were also required in the lytic phase raises the question whether the $CD4^+$ T cells in addition to their helper function are also cytotoxic. Also expected were cooperations between $CD4^+$ T, $CD8^+$ T and $CD1a^+CD83^+$ dendritic cells (**Fig. 5b,c,d**) which fit known models of mutual help and crosspriming. However, the absolute requirement of $CD14^+$ monocytes during the initial CD3-activation phase as well as during priming of naïve T cells comes as a surprise and suggests that *de novo* instruction and maturation of $CD14^+$ monocytes to dendritic cells is crucial. This instruction of $CD14^+$ monocytes could occur via activated $CD1a^+CD83^+$ dendritic cells, which differentiated naïve T cells to T effector cells and then received from freshly differentiated T effector cells the task to mature $CD14^+$ monocytes. It was recently reported that signals from activated $CD4^+$ T cells enable dendritic cells to instruct bystander dendritic cells to prime naïve $CD4^+$ T cells[41,42]. These instruction circuits apparently include bystanding $CD14^+$ monocytes.

During the lytic phase $CD14^+$ monocytes seem to play a minor role. Whereas depletion of dendritic cells immediately before coculture of CAPRI cells with cancer cells reduced cancer cell destruction by half, depletion of $CD14^+$ monocytes had only

minimal effects (P not significant) (**Fig. 5a,b**). This suggests that dendritic cells provide a continuous flow of cytokines and perhaps also of tumor-immunogenic information during the phase of cancer cell destruction by building a bridge of information between cancer cells and T cells. Thus maintaining the active effector status of T cells. This may be important only for cancer cells which show a low MHC expression and may explain, why depletion of dendritic cells reduces destruction only by half. Supplementary professional presentation by activated dendritic cells apparently prevents rudimentary TCR signalling by cancer cells leading, for example, to default secretion of suppressive IL-10 by Th1 cells[43].

CAPRI cells lysed a series of different autologous cancer cells (listed in **Table 1**, lysis not shown). Of particular note was the successful lysis of carcinoma in situ cells of Bowens disease by CAPRI cells. These intraepidermally growing carcinoma in situ cells are commonly recalcitrant to therapy because they are enveloped by fibroblasts. Less than one percent of the cells of the Bowenoid cell line bound keratinocyte antibodies in cytospins (not shown). This in situ cancer is an excellent example for the proposed inhibitory role of tumor stroma as this stroma usually prevents straightforward lysis by T cells[44]. We interpreted the lysis of Bowens in situ cancer cells by CAPRI cells as evidence for crosspresentation and crosspriming between members of the CAPRI cell quartett and perhaps as evidence for the ability of CAPRI cells to enhance fibroblast processing and presentation of tumor products, as has been described[45].

The observed reduction of $CD4^+CD25^{high}$ $FOXp3^+$ regulatory cells during the cascade priming (**Supplementary Fig. 1** online) fits present interpretations of $CD4^+CD25^{high}$ $FOXp3^+$ regulatory cells as suppressing anti-tumor activity. Hence, reduction of $CD4^+$ $CD25^{high}FOXp3^+$ cells would allow cytolytic activity of CAPRI cells. Whereas CD3-stimulation of T lymphocytes favours pathways leading to $CD25^{high}Foxp3^+$

expression[36], the natural activation via the αβ TCR[37] may favour amplification of $CD4^+$T cells, which do not express FOXp3.

The first case-controlled study with CD3-activated PBMC showed a small but significant reduction of recurrence and an increased survival rate in patients with hepatocellular carcinoma[46]. This result was interpreted as amplification of a small number of cancer specific T memory cells and not as result of CD3-activation of naïve T lymphocytes[47]. This interpretation would be compatible with our results of a marginal lysis of cancer cells by CD3-activated PBMC.

Adoptive immune cell therapies have often employed only one effector T cell type or one APC cell type to elicit an immune response against only one tumor-immunogenic peptide. These strategies should be reconsidered, since each population of the CAPRI cell quartett was pivotal in the functional circuits of priming or cancer cell lysis. Consequently, engineering peptides for helper and cytotoxic T cell responses would require design of tumor-immunogenic peptides which would be presented by HLA class I and HLA class II molecules. CAPRI cells increased/induced HLA class I and HLA class II expression in cancer cells and efficiently lysed different types of cancer (listed in **Table 1**). CAPRI-activated APC presenting tumor-immunogenic peptides could be isolated from the peripheral blood of cancer patients as suggested for immunogenic peptides from HIV resistant individuals[48].

Independent of these future aspects, CAPRI cells already now constitute a specific and powerful instrument in the fight against cancer. CAPRI cell-treated breast cancer patients of an intention-to-treat setting showed a highly significant improvement of survival rates after five years compared to breast cancer patients of the Munich tumor center (Laumbacher, Gu and Wank, unpublished results). A case-controlled study with CAPRI cells in non-small lung cancer patients has started in December 2007 at the University of Kunming in China.

Materials and methods

Tumor samples and establishment of autologous tumor cell lines

Immune cells and autologous tumor samples were donated by informed and consenting patients referred by doctors for support of radiation- or chemotherapy with adjuvant adoptive immunotherapy. Tumor samples were used to establish cancer cell lines to control lytic capacity of activated immune cells. The ethic recommendations of Helsinki with subsequent amendments of Tokyo 1975, Hong Kong 1989 and Somerset West 1996 were followed.

Tumor samples were minced to small pieces and cultured in 50ml-flasks using supplemented tumor culture medium (RPMI 1640 with L-glutamine, supplemented with 10% FCS, NEAA, G5 Supplement, all from PAA, Coelbe, Germany) and optimized culture conditions, unused tumor samples were also minced to small pieces and cryopreserved in DMSO like PBMC[49]. Establishment of cell lines with at least twenty cell divisions was successful only with samples from patients before chemo- or radiation therapy. The following cancer cell lines were used in most experiments: colon cancer line CRC5 was established from a patient´s liver metastasis, the melanoma cell line MEL12 from a recurring tumor (**Table 1**). Nine breast carcinoma lines as well as a bowenoid cell line were established from patients from southern Germany (**Table 1**). All cell lines originated from Caucasian patients.

Isolation of immune cells

PBMC were isolated from venous blood puncture or leukapheresis samples by density gradient centrifugation as described elsewhere[49] using lymphocyte separation medium (LSM, PAA, Coelbe, Germany). Immune cells were either used immediately

or cryopreserved and stored in the nitrogen gas phase. Isolation, cryopreservation and thawing procedures as well as the use of optimized culture conditions (38.5°C, 6.5% CO2) were described in detail[49].

Activation of T cells in PBMC bulk cultures: CD3-activation and CAPRI cell generation

Both methods started with activation of T cells in PBMC bulk cultures using the CD3 monoclonal antibody OKT3 (Orthoclone, Cilag, Sulzbach/Taunus, Germany) binding to the non-polymorphic ε-chain of the CD3 molecule and addition of Interleukin 2 (IL-2; Proleukin, Chiron, Ratingen, Germany).

CD3 antibodies were immobilized at a concentration of 1µg/ml, in 0.05M borate buffer pH 8.6 and distributed in 50ml tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany). Coated flasks were kept at 4°C (at least over night) and washed twice with phosphate buffered saline prior to incubation with PBMC. PBMC were added at a concentration of $2x10^6$ per ml in a total volume of 10ml, IL-2 was added within 2-12 hours at a concentration of 20U/ml. CD3-activated cells were expanded on day 4 with IL-2 (20U/ml) and harvested on day 7 for immediate use or cryopreservation.

For the generation of cascade primed (CAPRI cells) CD3-activated PBMC were removed from the flask after 4-6-hours, washed and cocultured in a second CD3 "antibody free" flask with an equal number of unstimulated autologous PBMC (at a concentration of $2x10^6$ per ml in a total volume of 10ml), which contained naïve/resting T cells. Cells were expanded on day 1 with IL-2 (20U/ml) and harvested on day 4.

Flow cytometry

Expression of cell surface markers was determined by flow cytometry using the Becton-Dickinson FACScan analyser and CellQuest software (Becton-Dickinson, Germany). $CD14^+$ cells were CFSE-labelled to trace changes in phenotype. In brief, cells were harvested and stained with anti-CD14 PE, HLA-DR-PE, CD1a-PECy5.5, with anti-CD40-PECy5.5, CD80-PECy5.5, CD83-PECy5, CD86-PECy5.5 and with anti-HLA-A,B,C-PECy5.5 for tracing the phenotype of CFSE labeled CD14 cells during CD3 or CAPRI stimulation. For acquisition of cell surface markers of CD3-stimulated and CAPRI cells, cells were collected and stained with anti-CD3-FITC, CD14-PE, CD19-PECy5.5 and with anti-CD3-FITC, CD4-PE, CD8-PECy5.5 and with anti-CD3-FITC, CD14-PE, CD56-PECy5.5 and with anti-CD3-FITC, CD16-PE, CD56-PECy5.5. For Foxp3 staining, cells were stained first with anti-CD4-PE, fixed, permeabilized with human Foxp3 staining buffer set and stained with FITC-anti-human Foxp3.

The conjugated mouse monoclonal antibodies were obtained from BD Biosciences or eBioscience. Human Foxp3 staining buffer set was obtained from eBioscience.

Separation of PBMC subpopulations with magnetic beads

Mouse anti–human CD3, CD4, CD8, CD14 conjugated to magnetic beads, CD14 negative isolation kits and Pan mouse IgG beads were obtained from Dynal (Invitrogen, Paisley, UK) and used according to manufacturer's instructions.

$CD4^+$ T cells were isolated from CD3 isolated populations to spare $CD14^+CD4^+$ monocytes.

Microscopic classification, preparation of tumor target cells and quantification of cancer cell destruction using the Cr ^{51} release assay

Cancer cells were removed from flasks by trypsinization, resuspended in 10% FCS enriched culture medium (RPMI 1640 with L-Glutamine, PAA, Coelbe, Germany) and washed twice. Cancer cells were counted and distributed in different concentrations in 96-well flat bottomed culture plates (Falcon, Becton Dickinson, Heidelberg, Germany) either for microscopic evaluation of lysis or for the Cr^{51} release assay. Cancer cells were allowed to recover over night to restore their membranes after trypsinization before addition of effector cells. Reorganization of cancer cells into a coherent cell layer better reflected the situation *in vivo* and significantly lowered the rate of spontaneous release of Cr^{51}, a so far unresolved problem of the Cr^{51} release assay in measuring tumor cell destruction.

The Cr^{51} release assay was performed in duplicates at varying effector : target ratios, using 2 x 10^3 cancer cells as targets. Maximum release was determined using labelled target cells, spontaneous release by incubating target cells in medium alone. Percent spontaneous release was calculated as follows: (spontaneous cpm : maximum cpm) x 100; and the percent cytotoxicity was calculated as follows: [(experimental cpm – spontaneous cpm) : (maximum cpm – sponatanious cpm)] x 100. Quantitative lysis of cancer cells using the Cr ^{51} release test was assessed after 5-6h and after 18-22h, following the "classical" guidelines of the CML assay[23] with the crucial difference of tumor target preparation described above.

Estimation of the degree of lysis was performed by microscopic inspection after 18-24h. The scale used corresponded to conventional HLA microscopic estimated evaluation, designating more than 80% cell lysis as strong positive (++) and 60-79% as positive; 40-59% as weak positive, 20-39% (+) as doubtful positive and less than 20% (-) as negative (**Fig 2**).

To determine the influence of HLA class I and class II molecules on cancer lysis, monoclonal antibodies were added at the start of the immune cell - cancer cell cocultures. The antibody W6/32 (abcam, Cambridge, UK) was used to block HLA class I (1μg/ml) and HLA workshop-defined anti DQ2 antibody C1A2 (1μg/ml) (11WO938; WAN 806, kindly provided by Prof. J.P. Johnson, Munich) was used to block HLA class II.

Statistical analysis

The slope and y intercept of regression lines obtained from CML titrations were evaluated using the General Linear Model (GLM) procedure. The statistical package SPSS 10.1 (SPSS Inc., Chicago, Illinois) was used.

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Authors contribution

Barbara Laumbacher and Rudolf Wank pioneered the CAPRI cell procedure over several years. Songhai Gu designed and performed the elegant FACS experiments. All authors participated in writing the manuscript.

Legends to figures

Figure 1

Comparison of cancer cell lysis by CAPRI cells and by CD3-activated PBMC.

Breast cancer cells (**a,b**), CD3-activated PBMC (**c**) and CAPRI cells (**d**) were subsequently cocultured (**e,f**). The degree of lysis by CD3-activated PBMC (**e**) or CAPRI cells (**f**) was compared microscopically (**c,d**) and by the chromium^{51} release test (**g**) after 20h. Effector (E) to target (T) ratio was 20:1 (**a,b,c,d,e,f,g**) titrated to 5:1 in the chromium^{51} release assay (**g**). The number of cancer cells appeared unchanged with cocultured CD3-activated PBMC after 20h (**e**), whereas CAPRI cells clearly reduced the number of cancer cells (**f**) as demonstrated quantitatively using Cr^{51} labelled cancer cells as targets (**g**).

Our modification of the CML (cell mediated lympholysis) test[23] kept the spontaneous release under 10% during assay periods of 18-24h.

Figure 2

CAPRI cells lyse cancer cells MHC restricted. MHC restricted lysis by CAPRI cells, using a E:T ratio of 20:1, was determined microscopically after 24h in two ways: (**a**) by using autologous cancer cells and cancer cells sharing or not sharing HLA-DQ alleles, (**b-f**) by presenting lysis and HLA-blocked lysis of autologous cancer cells. (**a**) autologous cancer cells were lysed the strongest (++); cancer cells sharing HLA-DQB1*0603 or DQB1*0201 with CAPRI-CTL1 (upper line, HLA-DQB1*0201 and DQB1*0202 express identical surface molecules), or sharing HLA-DQB1*0604 or DQB1*0301 with CAPRI-CTL2 were noticeably lysed (+). No significant lysis occurred without HLA-DQ class II matching; the quantity of lysed cancer cells was estimated analogous to the HLA microcytotoxicity test. (**b**) autologous breast cancer

cells without CAPRI cells, (**c**) lysis of autologous cancer by CAPRI cells, (**d**) lysis blocked by HLA-DQ class II antibody, (**e**) lysis blocked by HLA class I antibody, (**f**) no blocking of lysis by isotype control antibody.

Figure 3

Increased HLA class I and HLA class II expression of breast cancer cells after cocultivation with CAPRI cells. To save sufficient unlysed cancer cells for observation a low effector:target (E:T) ratio of 5:1 was used. HLA expression of CFSE-labelled colon cancer cells F2 was determined after five days of coculture with (**a**) CAPRI cells, (**b**) CD3-activated PBMC or (**c**) unstimulated PBMC. CAPRI cells stimulated cancer cells to a mean increased fluorescence (MIF) expression of HLA class I by 40% and of HLA-DR class II antigens by 60% (**a**). No significant change of HLA expression of cancer cells could be observed after incubation with CD3-activated (**b**) or unstimulated PBMC (**c**).

Figure 4

Expression of differentiation and activation markers by CFSE labelled $CD14^+$ monocytes during cascade priming and CD3-activation of PBMC. PBMC with reimported CFSE-labelled $CD14^+$ monocytes were split in three groups: PBMC were left unstimulated (**a**), submitted to cascade priming (**b**) or CD3-activated (**c**). Developmental and functional stages of labelled monocytes were characterized with specific antibodies recognizing CD14 (monocytes), CD83 and CD1a (dendritic cells), CD80, CD86, CD40 (activation markers) and HLA-DR class II and HLA class I molecules at days 0 (**a**), 1 (**b,c**) and 5 (**b,c**). Whereas the number of $CD14^+$ monocytes declined in cascade primed cells cultures at days 1 and stronger at day 5 (**b**), numbers of $CD14^+$ monocytes in unstimulated (**a**) and in CD3-activated PBMC

(**c**) remained essentially unchanged at days 1 and 5. In contrast, numbers of $CD83^+$ and $CD1a^+$ cells increased in cascade primed cell cultures (**b**) but not in CD3-activated PBMC and unstimulated PBMC (**a,c**). Increased expression of activation markers CD80, CD86, CD40 and HLA-DR class II and HLA class I molecules was clearly stronger in cascade primed cell cultures (**b**) than in CD3-activated PBMC and unstimulated PBMC (**a,c**).

Figure 5

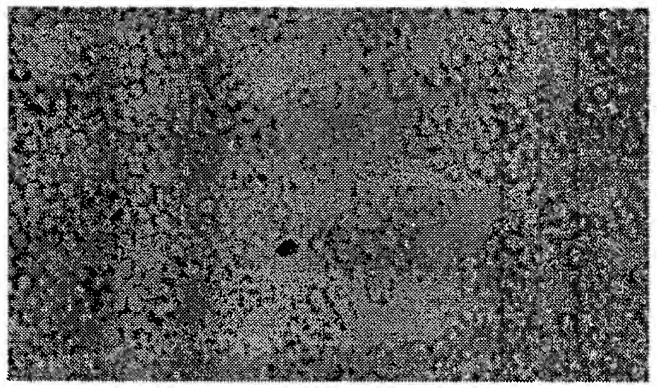
Interdependence of monocytes, dendritic cells, T helper and T cytotoxic cells for priming and cancer cell lysis.

The contribution of immune cell populations to the cytotoxic capacity of CAPRI cells against breast cancer cells was determined at different phases of activation and cancer cell destruction (**a**) by depleting PBMC from $CD14^+$ monocytes, (**b**) from $CD83^+$ dendritic cells, (**c**) from $CD3^+CD4^+$ T helper cells, or (**d**) from $CD3^+CD8^+$ T cytoxic cells. Depletion occurred at the following points in time: before CD3-activation of PBMC (···◇···), from unstimulated PBMC to be added to CD3-activated PBMC for T cell priming (···△···), from CAPRI cells before incubation with cancer cells (···●···). Not depleted CAPRI cells (—■—) ensured validity of tests (**a,b,c,d**). (**a**) Presence of $CD14^+$ monocytes was absolutely required for development of cytotoxicity in the priming procedure, during CD3 activation of PBMC and during priming of PBMC with CD3-acivated PBMC ($P_{slope} = 0.003$, $P_{intercept} = 0.001$, for both PBMC populations) and had minimal non-significant impact on the lytic power of CAPRI cells *($P_{slope} = 0.37$, $P_{intercept} = 0.057$)*. (**b**) Depletion of $CD83^+$ dendritic cells reduced development of cytotoxicity during CD3-activation of PBMC *($P_{slope} = 0.00072$, $P_{intercept} = 0.008$)*, during priming of PBMC with CD3-acivated PBMC ($P_{slope} = 0.004$, $P_{intercept} = 0.007$) and reduced also the lytic power of CAPRI cells significantly *($P_{slope} = 0.01$, $P_{intercept} =$*

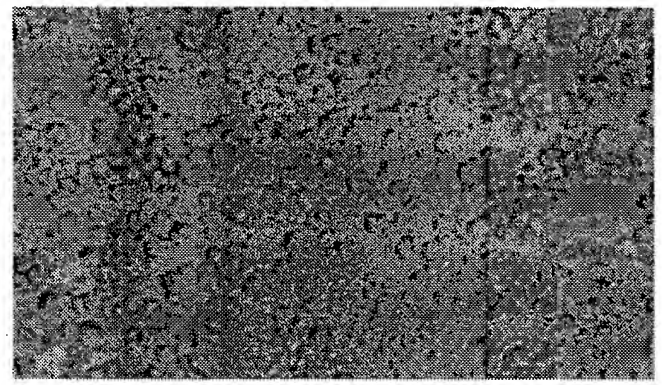
0.004*)*. There was no significant difference between the dendritic cell depletions at the three points of time. (**c**) $CD3^+$ $CD4^+$ T helper cells were absolutely required during the CD3-activation of PBMC ($P_{slope} = 1.25 \times 10^{-7}$, $P_{intercept} = 0.022$), during priming of PBMC with CD3-acivated PBMC ($P_{slope} = 1.59 \times 10^{-6}$, $P_{intercept} = 0.015$) and for cytotoxicity of CAPRI cells ($P_{slope} = 1.59 \times 10^{-6}$, $P_{intercept} = 0.015$) . (**d**) $CD8^+$ T cytoxic cells had a key role in all phases of the cascade priming procedure and cancer cell lysis ($P_{slope} = 0.003$, $P_{intercept} = 0.001$, at all three points of time).

Figure 1

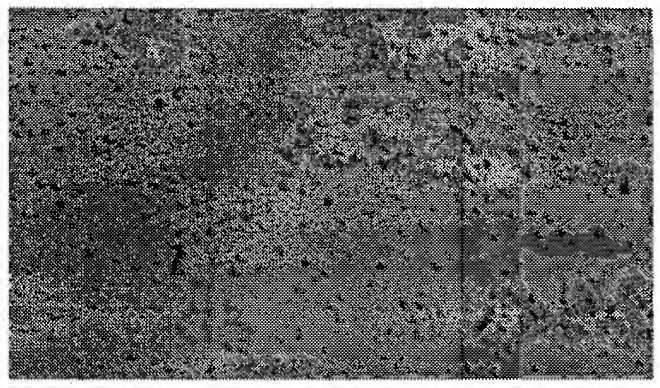
a) breast cancer cells



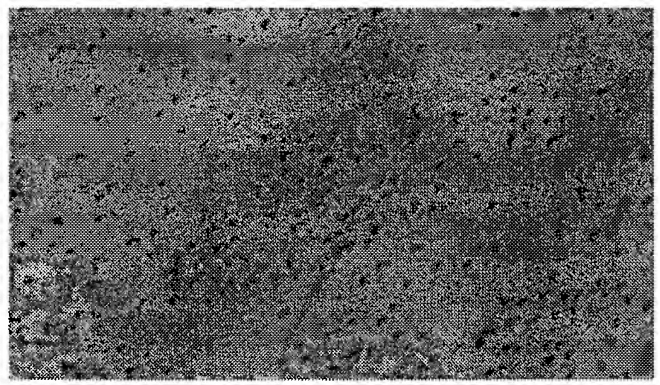
b) breast cancer cells



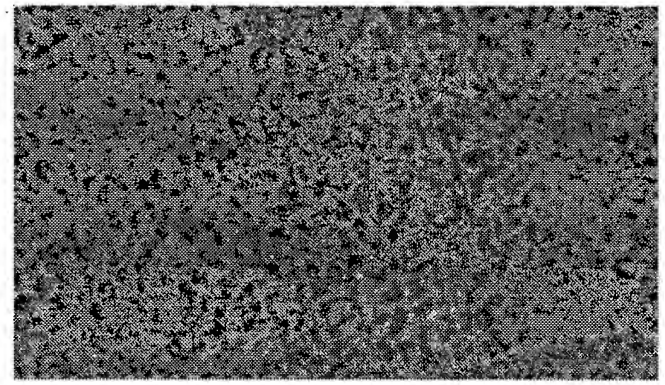
c) CD3-activated PBMC



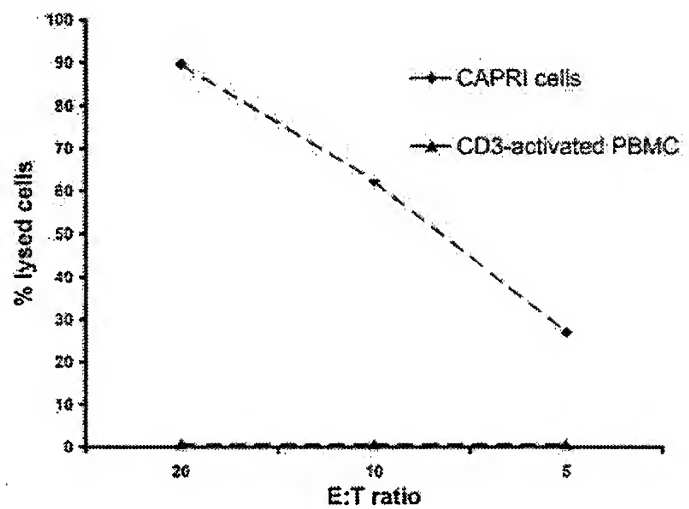
d) CAPRI cells



e) breast cancer cells + CD3-activated PBMC after 20hs of coculture



f) breast cancer cells + CAPRI cells after 20hs of coculture



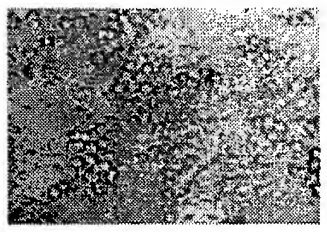
g)

Figure 2

a

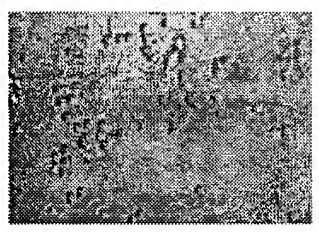
CAPRI cells		HLA-DQB1 alleles of breast cancer cell lines, used as targets									
HLA-DQB1	autologous breast cancer cell lines	***0201** ***0201**	*0602 ***0201**	***0201** *0202	*0603 ***0201**	*0302 *0202	***0603** ***0603**	****0301*** ****0301***	*0501 *0501	****0604*** ****0301***	*0501 *0602
CAPRI 1 ***0603** ***0201**	++	+	+	+	+	+	+	-	-	-	-
CAPRI 2 ****0604*** ****0301***	++	-	-	-	-	-	-	+	-	+	-

b



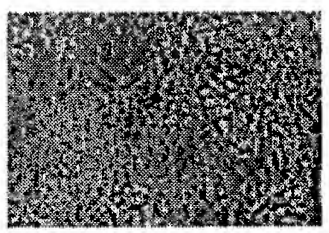
Breast cancer cells, without CAPRI cells

c



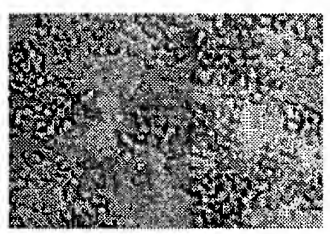
Breast cancer cells and CAPRI cells (positive control)

d



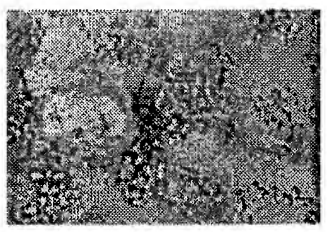
Breast cancer cells, CAPRI cells and HLA-DQ antibody

e



Breast cancer cells, CAPRI cells and HLA class I antibody (W6/32)

f



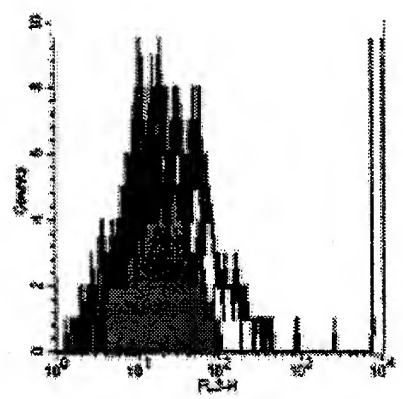
Breast cancer cells, CAPRI cells and irrelevant antibody (isotype control)

Figure 3

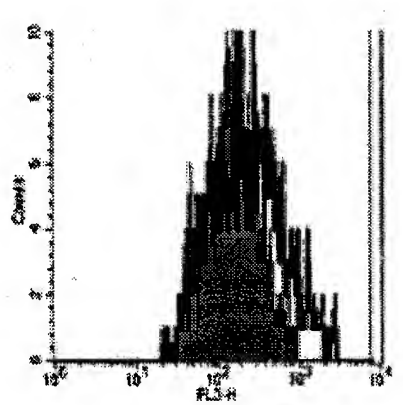
HLA-class I expression of carcinoma cells

HLA-DR class II expression of carcinoma cells

a



Incubation with CAPRI cells



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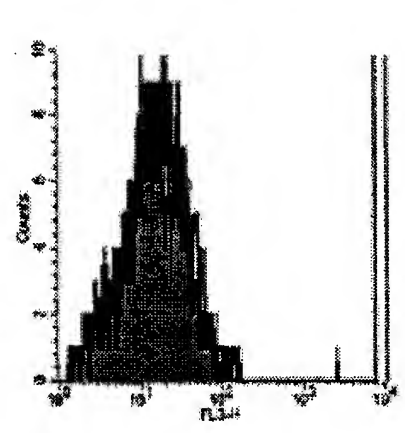
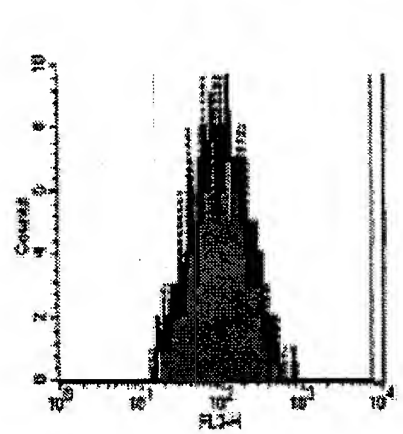


Incubation with CD3-activated PBMC



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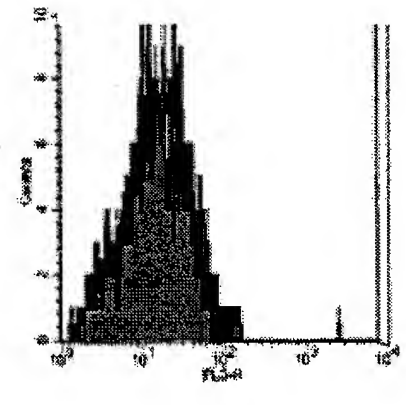


Incubation with PBMC

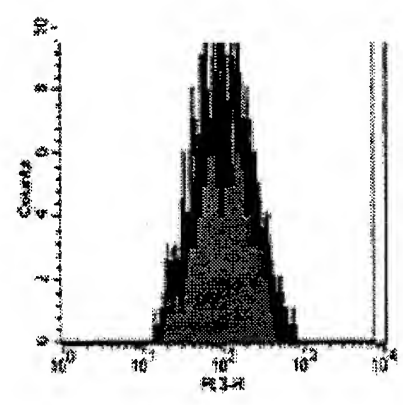


Figure 4

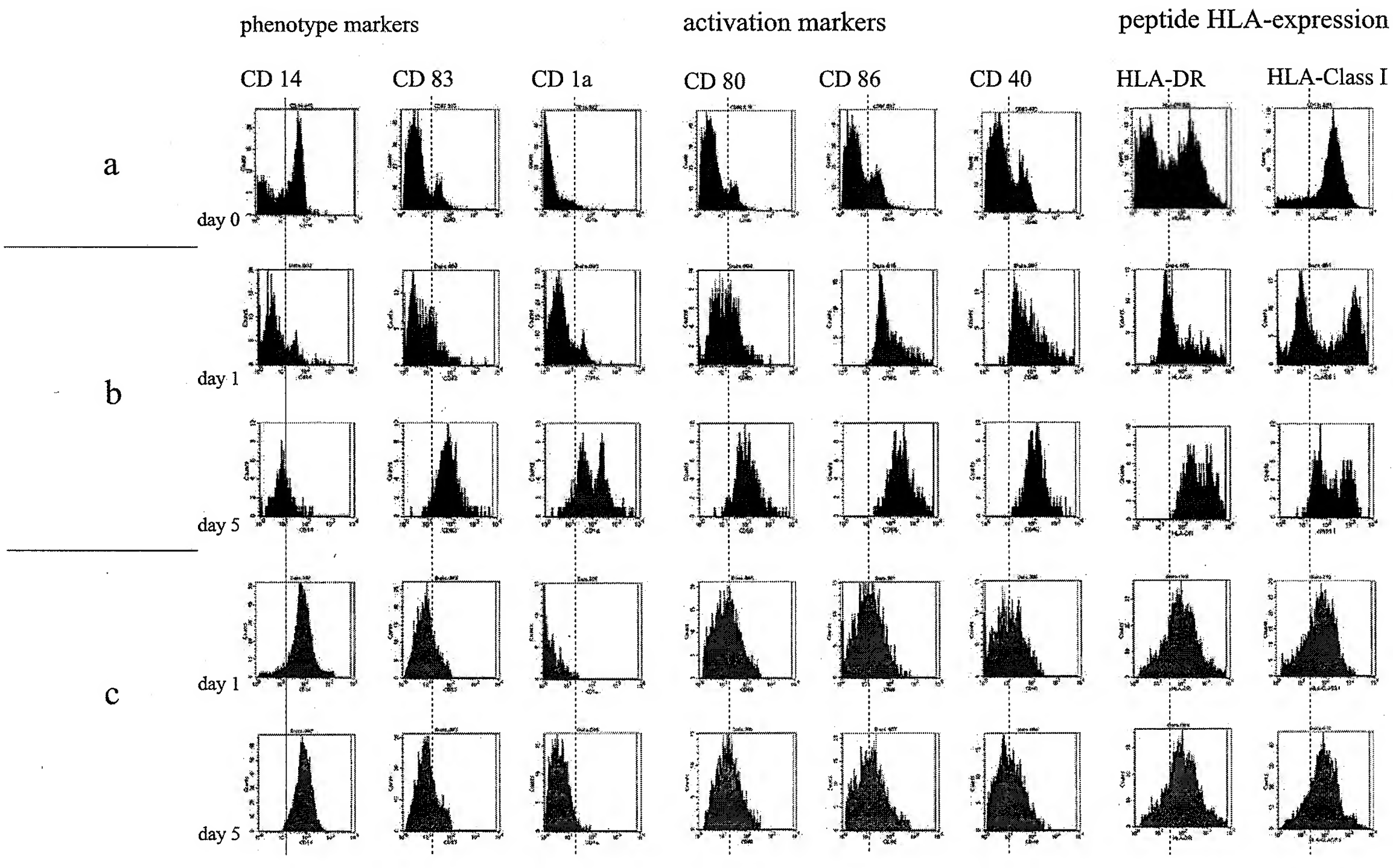
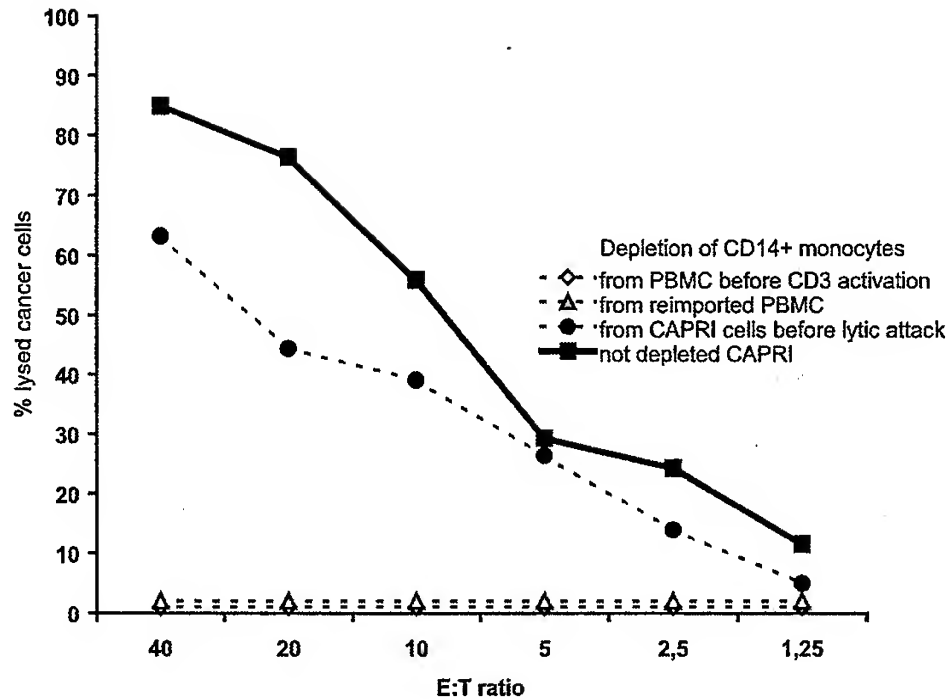

phenotype markers
activation markers
peptide HLA-expression
CD 14
CD 83
CD 1a
CD 80
CD 86
CD 40
HLA-DR
HLA-Class I
a
day 0
day 1
b
day 5
day 1
C
day 5

Figure 5

a



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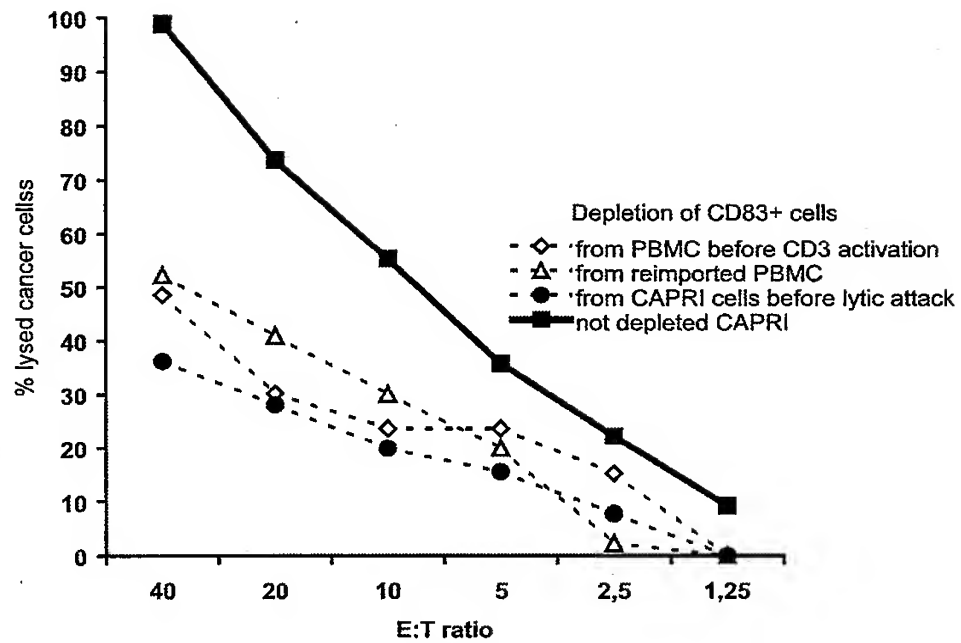

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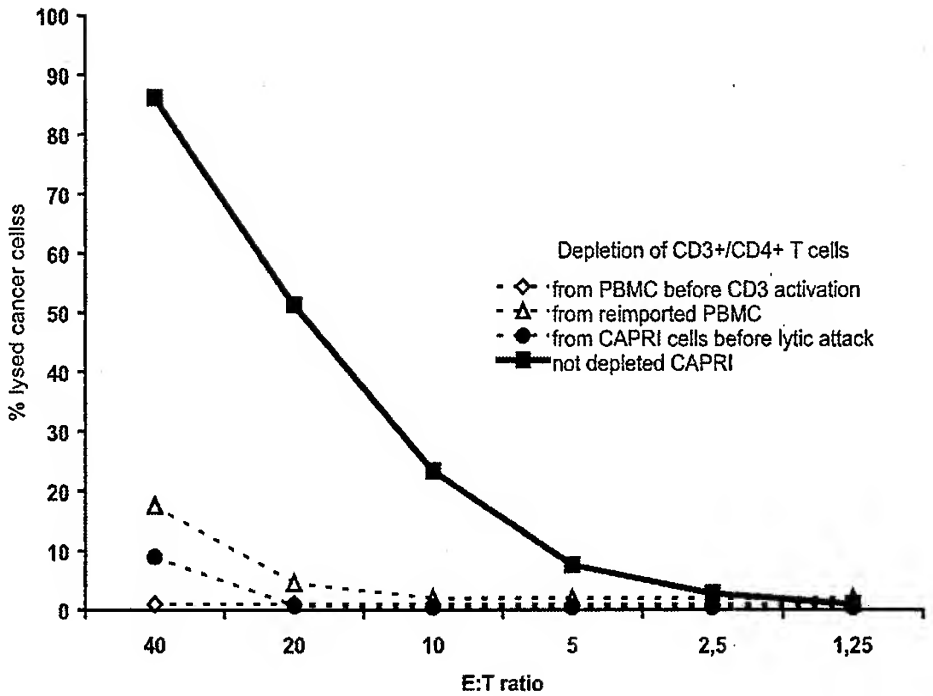

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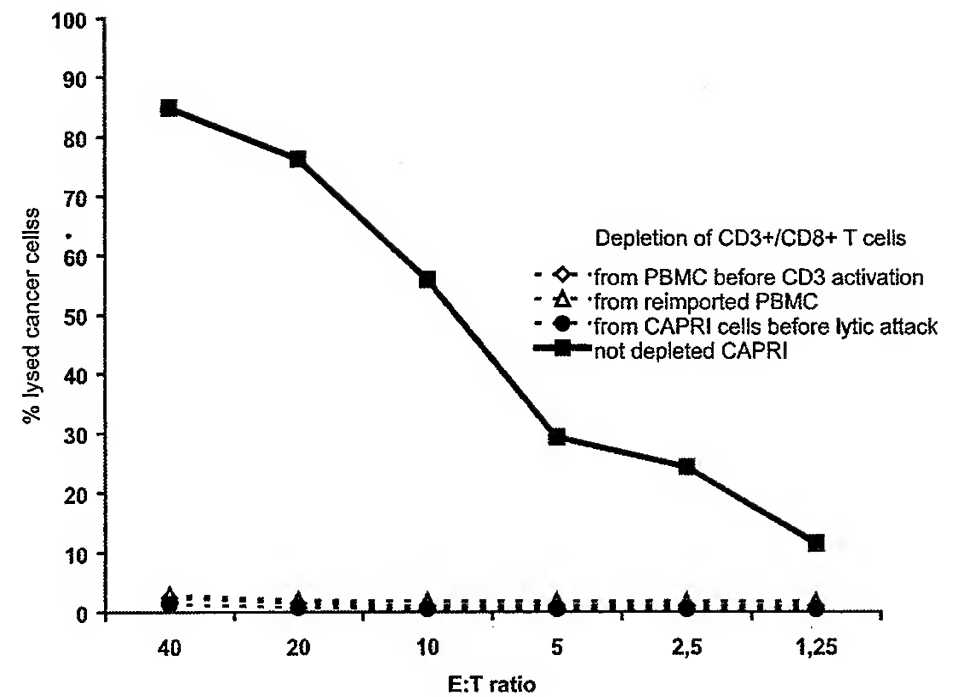
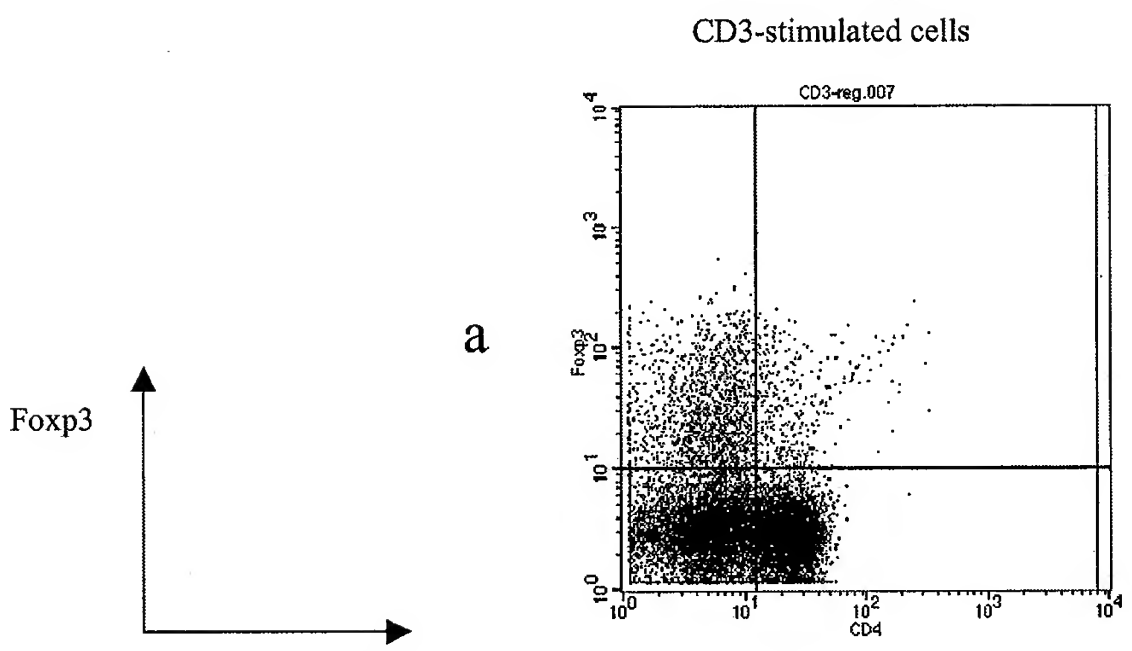


Supplementary figure 1



Supplementary Figure Legend 1

CD3 stimulated cells (fig. 1a) and CAPRI cells (fig,1b) were analyzed for Foxp3 expression in $CD4^+$ cells. Fewer regulatory T helper cells ($CD4^+/Foxp3^+$) were found in CAPRI cells (1.47%) than in CD3 stimulated cells (3.58%).

Supplementary Table 1

Lymphocyte subpopulations (percentage +/- standard.deviation) of CD3-stimulated and CAPRI cells of five cancer patients.

	CD3	CAPRI
$CD14^+$	2.63±1.30	0.44±0.08[a]
$CD19^+$	3.85±1.78	4.08±2.05
$Lin^-CD83^+HLA\text{-}DR^+$	0.52±0.29	2.14±0.41[b]
$CD3^+$	88.27±4.17	87.87±3.81
$CD3^+CD4^+CD8^-$	42.75±19.99	51.35±14.70
$CD3^+CD4^-CD8^+$	42.43±18.08	35.55±10.71
$CD3^+CD4^-CD8^-$	10.93±5.12	8.50±4.48
$CD3^+CD4^+CD8^+$	3.62±2.23	4.66±2.97
$CD3^+CD14^-CD56^+$	25.60±3.49	28.17±6.10
$CD3^-CD56^+$ or $CD16^+$)	4.73±2.01	5.15±2.60
$CD3^-CD16^+CD56^-$	0.18±0.25	1.04±1.99
$CD3^-CD16^-CD56^+$	3.64±2.35	2.74±0.92
$CD3^-CD16^+CD56^+$	0.90±0.33	1.36±0.29

CD3 stimulated cells contained a higher percentage of monocytes (paired *t* test, *P*=0.023), but a lower percentage of matured dendritic cells (paired *t* test, *P*=0.000096) than Capri cells. No other differences were observed between the cell subpopulations of CD3-stimulated and CAPRI cells.

Supplementary Figure Legend 1

CD3 stimulated cells (fig. 1a) and CAPRI cells (fig,1b) were analyzed for Foxp3 expression in $CD4^+$ cells. Fewer regulatory T helper cells ($CD4^+/Foxp3^+$) were found in CAPRI cells (1.47%) than in CD3 stimulated cells (3.58%).

CD3 stimulated cells (fig. 1a) and Capri cells, fig,1b) were analyzed for Foxp3 expression in $CD4^+$ and $CD4^-$ cells. Fewer regulatory T helper cells ($CD4^+/Foxp3^+$) were found in CAPRI cells (1.47%) than in CD3 stimulated cells (3.58%). Foxp3 expression was also lower in $CD4^-$ cells (most of them were $CD8^+$) in CAPRI preparation (3.22%) compared to CD3-stimulated cells (11.05%). The $CD4^-(CD8^+)/Foxp3^+$ T cells were supposed to overlap with $CD8^+/CD25^+/CTLA4^+/Foxp3^+$ regulatory $CD8^+$ T cells.

associate with β-2 microglobulin) that are recognized by the lectin-like receptor NKG2D. The MIC-A and -B proteins are inducible and are expressed on cells that have been stressed by infection, heat, or trauma. When the lectin-like NKG2D receptor binds to these stress-induced proteins, the response includes recruitment of cytotoxic granules and release of cytokines, leading to the death of the targeted cell. Other activating receptors present on most NK cells include NKp30, NKp44, and NKp46. These structurally related Ig-like molecules are involved in killing of tumor cells, but the identities of the tumor-cell ligands they recognize are not known.

NK-cell inhibitory ligands are better characterized than activating ligands. Clues to the sources of inhibitory signals came from studies of the killing of tumor cells and virus-infected cells by NK cells. Researchers determined that the preferential killing of mouse tumor cells compared with normal cells correlated with a lack of expression of MHC molecules by the tumor cells. Experiments with human cells showed that NK cells lysed a B-cell line that was MHC deficient as a result of transformation by Epstein-Barr virus. After this cell line was transfected with human HLA genes, which caused it to express high levels of MHC molecules, NK cells failed to lyse it. These observations led to the idea that NK cells target for killing those cells that have aberrant MHC expression. Since many virus-infected and tumor cells exhibit diminished MHC expression, this model, called the "missing self model," made good physiological sense. Support for this proposal has come from the discovery of receptors on NK cells that produce inhibitory signals when they recognize MHC molecules on potential target cells. These inhibitory receptors on the NK cell then prevent NK-cell killing, proliferation, and cytokine release.

In humans, most inhibitory receptors are Ig-like molecules. An exception is the lectin-like inhibitory receptor CD94/NKG2A, a disulfide-bonded heterodimer made up of two glycoproteins, CD94 and a member of the NKG2 family. The CD94/NKG2A receptors recognize HLA-E on potential target cells. Because HLA-E is not transported to the surface of a cell unless it has bound a peptide derived from HLA-A, HLA-B, or HLA-C, the amount of HLA-E on the surface serves as an indicator of the overall level of class I MHC biosynthesis in the cells. These inhibitory CD94/NKG2A receptors recognize the surface HLA-E and send inhibitory signals to the NK cell, with the net result that killing of potential target cells is inhibited if they are expressing adequate levels of class I.

KIR inhibitory receptors, which show considerable diversity, are generally specific for a single polymorphic product of a particular HLA locus or for a limited number of related HLA molecules. Unlike antibodies in B cells and TCRs in T cells, which have the property of allelic exclusion, NK cells are not limited to expressing a single inhibitory KIR but may express several, each specific for a different MHC molecule or for a set of closely related MHC molecules. For example, individual ones of human NK cells expressing a CD94/NKG2A receptor had as many as six different KIR receptors have been found.

Because signals from inhibitory receptors can counteract signals from activating receptors, a negative signal from any inhibitory receptor, whether of the CD94/NKG2A or KIR type, can block the lysis of target cells by NK cells. Thus, cells expressing normal levels of unaltered MHC class I molecules tend to escape all forms of NK-cell-mediated killing. Surprisingly, the KIR family appears to have evolved extremely rapidly. Functional KIR receptors found in the primate line do not exist in rodents. Mice use a different family of receptors, the lectin-like Ly49 family, to achieve the function of KIRs, namely inhibition of NK cells through binding to MHC class I molecules on healthy cells. Functional Ly49 receptors do not exist in humans.

In the opposing-signals model of NK-cell regulation that is emerging from studies of NK cells (Figure 14-14), activating receptors engage ligands (most of which are not known) on the surface of a targeted tumorous, virus-infected, or otherwise stressed cell. Recognition of these determinants by activating receptors would signal NK cells to kill the target cells. Killing signals can be overridden by signals from inhibitory receptors. As we have already seen, the inhibitory receptors provide a signal that decisively overrides activation signals when these inhibitory receptors detect normal levels of MHC class I expression on potential target cells. This prevents the death of the target cell and also NK-cell proliferation and the induction of secretion of cytokines such as IFN-γ and TNF-α. The overall consequence of the opposing-signals model is to spare cells that

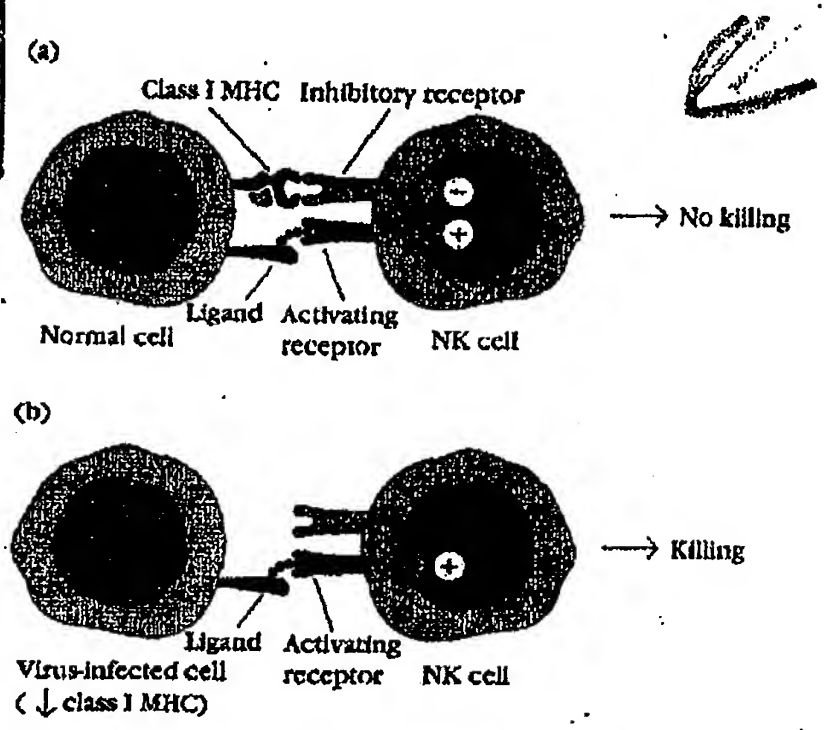


FIGURE 14-14 Opposing-signals model of how cytotoxic activity of NK cells is restricted to altered self cells. An activating receptor on NK cells interacts with its ligand on normal and altered self cells, inducing an activation signal that results in killing. However, engagement of inhibitory NK-cell receptors such as inhibitory KIRs and CD94/NKG2 by class I MHC molecules delivers an inhibition signal that counteracts the activation signal. Expression of class I molecules on normal cells thus prevents their destruction by NK cells. Because class I expression is often decreased on altered self cells, the killing signal predominates, leading to their destruction.

Kuby Immunology, 6th edition (2006), W.H. Freeman and Company, p. 363